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Flight Performance and Teneral Energy Reserves of Two Genetically-Modified and One Wild-Type Strain of the Yellow Fever Mosquito *Aedes aegypti*

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Abstract

The ability of sterile males to survive, disperse, find, and mate with wild females is key to the success of sterile insect technique (SIT). The Release of Insects carrying a Dominant Lethal (RIDL) system is a genetics-based SIT strategy for *Aedes aegypti*. We examine two aspects of insect performance, flight potential (dispersal ability) and teneral energy reserves, by comparing wild-type (WT) males with genetically-modified lines carrying the tetracycline-repressible constructs OX513A and OX3604C. Our results show significant differences in the flight capacity of the modified lines. OX513A males bred with tetracycline covered 38% less distance, while OX3604C males reared without tetracycline spent 21% less time in flight than their WT counterparts. Such differences in flight performance should be considered when designing release programs (e.g., by placing release sites sufficiently close together to achieve adequate coverage). All mosquito lines had similar teneral carbohydrate contents, though males of the OX3604C line contained more lipids. The addition of tetracycline to the larval diet did not influence the flight potential of the males; however, it did change the teneral sugar reserves of the WT and the lipid reserves of both the WT and the OX3604C lines.

Key Words: *Aedes aegypti*—Sterile Insect Technology—Tetracycline—Yellow Fever.

Introduction

The *Aedes aegypti* mosquito is the most important vector of dengue fever, and has therefore been the target of various vector control programs (Reiter and Gubler 1997), and advances in genetic transformation technology have allowed the development of new approaches to its control. One of these is the release of insects carrying a dominant lethal gene (Release of Insects carrying a Dominant Lethal, RIDL®; Alphey et al. 2002, 2008, 2010; Thomas 2000), a genetics-based control system modeled on the traditional sterile insect technique (SIT). RIDL mosquitoes are engineered with tetracycline-dependent repression (Phuc et al. 2007; Fu et al. 2010), as tetracycline can be introduced as a dietary supplement for mosquitoes reared in the laboratory, but is not readily available in the wild. Thus the lethal system can be repressed in the laboratory and is activated upon release. The transformed males, which are homozygous for the engineered construct, pass one copy of the dominant lethal gene to their offspring. In the OX513A line, these die as larvae or pupae in the wild due to the absence of tetracycline; in the OX3604C line females emerge but are unable to fly (Fu 2010). Therefore, releases of modified males should reduce, and may under appropriate circumstances eliminate, the targeted mosquito population (Phuc et al. 2007; Atkinson et al. 2007; Wise de Valdez et al. 2011). As the control strategy can work only if the engineered males can pass on their genes, the ability of released males to successfully compete for females in the field is a critical aspect of its success.

The flight potential of *A. aegypti* is a possible proxy for mating success, as flight plays an important role in the mating biology of this species. Mating often takes place at or near the human host (Teesdale 1955; Hartberg 1971), so insects must disperse from their breeding site to find a suitable mating area. Furthermore, flight plays an important role during mating; males initiate mating in flight after identifying the

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female by sound (Roth 1948), at which point the synchronization of wing beat frequencies plays a key role in their mating success (Gibson and Russell 2006; Cator et al. 2009). Therefore differences in the flight performance of males may influence their mating success.

The energy reserves necessary to perform these tasks includes a combination of teneral reserves (energy available upon emergence), and reserves built up through feeding after emergence. Moreover, maximal flight performance has been shown to be linked to the available energetic reserves of a mosquito (Briegel et al. 2001a, 2001b; Kaufmann and Briegel 2004), and therefore may represent an indicator of overall fitness. This article compares the flight performance and the teneral energy reserves of males of two RIDL lines (OX513A and OX3604C) and a wild-type (WT) counterpart of *A. aegypti*.

In addition, we examine the effect adding tetracycline to the larval diet has on the flight potential and energy reserves of males of the WT and OX3604C lines (OX513A cannot be reared without tetracycline). Tetracycline has been used to eliminate symbionts in a variety of insects, as it kills bacteria by inhibiting protein synthesis (Goldman et al 1983; Speer at al. 1992; Chopra and Roberts 2001). Few studies exist that examine the effect tetracycline may have on insect performance, though Thompson and Sikorowski (1984) showed that the addition of tetracycline had a detrimental impact on the performance of *Heliothis virescens* when added to the larval diet.

Materials and Methods

All experiments were conducted in a temperature-controlled insectary at 28 ± 2°C and a relative humidity of 65 ± 10% with a 12-h:12-h light/dark cycle.

Mosquito lines

Wild-type line (WT). The WT line originated from field-caught *Aedes aegypti* from Jinjang, Selangor, Malaysia. It was colonized in 1975 and has been held in the laboratory for many generations. Although it is a lab-adapted strain and therefore not fully representative of males in natural populations, we used it because of its genetic similarity to the modified RIDL lines (see below).

The bi-sex lethal line (OX513A). OX513A is a homozygous RIDL line of *A. aegypti*, transformed with a tetracycline-repressible lethal positive feedback system (Phuc et al. 2007). A tetracycline-repressible transcriptional transactivator (tTAV; Gossen and Bujard, 1992; Gong et al. 2005), under the control of its own binding site (tetO), creates a positive feedback loop. Consequently, in the absence of tetracycline, tTAV accumulates to such high levels in cells of OX513A transgenic mosquitoes to cause lethality as late larvae or pupae, possibly due to the known interaction of the VP16 domain with key transcription factors (transcriptional squelching) (Baron et al. 1997; Phuc et al. 2007; Lin et al. 2007). The addition of tetracycline, on the other hand, leads tTAV to bind tetracycline, in which form tTAV can no longer bind to tetO and the cycle is interrupted (Phuc et al. 2007). Mosquitoes of this line are identifiable by red fluorescence due to the expression of DsRed2 under the control of a promoter fragment from the *Drosophila melanogaster Act5C* gene (Phuc et al. 2007).

The OX513A line was originally created in the Rockefeller strain and subsequently out-crossed into a Mexican line of *A. aegypti* (Harris et al. 2011). It has since been crossed to the WT line described above such that at least 97–99% of their genomes should be identical.

The female-specific flightless line (OX3604C). OX3604C is a homozygous line of *A. aegypti* transformed with a tetracycline-repressible sex-specific system that produces a flightless phenotype in females (Fu et al. 2010). The production of tTAV is controlled by the *AeAct-4* promoter in combination with a sex-specifically modified alternative splicing region, targeting gene expression only in the indirect flight muscles of females. Therefore, males can develop normally without the addition of tetracycline to their larval diet. Mosquitoes of this line are identifiable by all-over-body red fluorescence due to the expression of hr5ie1-DsRed2, and blue eyes due to the expression of 3xP3-AmCyan (Fu et al. 2010).

This line was created in the WT line and thus has a similar genetic background.

Larval rearing

Mosquito eggs (WT, OX513A, and OX3604C) were submerged in water and subjected to low pressure for 1 h to ensure synchronous hatching. The following day, the larvae were placed individually into the wells of 12-well microtiter plates containing 3 mL of water and fed the following feeding regimen of finely ground TetraMin® fish food per individual larva: day 1, 0.06 mg; day 2, 0.12 mg; day 3, 0.24 mg; day 4, 0.36 mg; day 5, 0.48 mg; days 6 and later, 0.6 mg. This rearing regimen was chosen because preliminary trials produced adult mosquitoes of similar size.

WT and OX3604C larvae were reared with or without tetracycline (tet) added to their rearing water at a concentration of 30 μg/mL, whereas OX513A (which requires tet for survival; Phuc et al. 2007) was reared solely on tet.

To ensure uniformity, only mosquitoes that pupated 7 days and emerged 9 days after hatching were used (> 80% of larvae pupated on day 7). Mosquitoes used in the flight mill trials and for wing length measurements were kept in large holding cages for 3 days and provided a 10% sucrose solution before being used; the mosquitoes used in the biochemical analyses were killed (briefly frozen) shortly after eclosing (the cages were checked every 2 h). For the biochemical analyses, males were fixed in absolute ethanol, which was removed by evaporation over a water bath at 90°C, and were subsequently stored at room temperature for less than 2 weeks before analysis.

Wing length measurements

The wings were removed in a 70% ethanol solution under a dissection microscope and mounted on microscope slides. Digital images of the wings were taken with a Canon PowerShot S5IS camera and a 99-mm adapter. The wings were measured with ImageJ softare (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe on 1-day-old males.

Flight mill system

The flight mills were constructed according to the design described by Rowley and associates (1968; flight path circumference 32.7 cm). As males do not actively mate in the first

24 h following eclosion (Roth, 1948), and peak flight activity of sucrose-fed females was demonstrated from 3 days post-eclosion onward (Briegel et al. 2001a), 3-day-old males were used. They were mounted on one arm of the flight mills with heated wax, and their flight was recorded by registering the number of revolutions at 30-sec intervals (Briegel et al. 2001a, 2001b). Flight trials were set up daily at approximately 10 AM and ran for 20 h, providing data on the total flight distance, the temporal pattern of flight activities (i.e., bursts of continuous flight or erratic flight pulses), and resting periods for each male tested.

In all, 269 flight trials were conducted (55 WT off tet, 47 WT on tet, 61 OX3604C off tet, 48 OX3604C on tet, and 58 OX513A on tet), of which we analyzed the 99 trials yielding useful data (i.e., during which the males had flown more than 500 meters; 21 WT off tet, 14 WT on tet, 26 OX3604C off tet, 14 OX3604C on tet, and 24 OX513A on tet). Trials with less than 500 meters of recorded flight activity were rejected, because it is likely the mosquitoes were poorly mounted on the flight mills in these cases (Kaufman and Briegel 2004).

Biochemical analyses

To quantify teneral nutrient reserves (lipid, glycogen, and sugar), 25 males of each line and rearing treatment were analyzed individually. Total lipid, glycogen, and sugar contents were measured with methods described by Van Handel (1985a, 1985b). Lipid content was quantified with a vanillin-phosphoric acid reaction (3 mL vanillin per tube), with 0.1% soybean oil in chloroform as a standard. Glycogen in the precipitate and sugar in the aqueous fraction were measured with a hot anthrone reaction (3 mL anthrone/tube), with glucose standards (0.1% in 25% ethanol). Absorbance values for 100 μL/well of processed experimental males and standard samples were measured in 96-well plates by a microplate reader at $\lambda = 630$ or 490 nm for carbohydrate and lipid, respectively, and further converted to micrograms per male with a regression line derived from the standard sample values. To compare energy content, the metabolites were converted to joules (J).

Statistical analysis

Statistical analyses were performed with JMP version 7.0 (http://www.jmpdiscovery.com). The flight parameters, energy reserves, and wing lengths of the three lines were compared using analyses of variance (ANOVAs). Flight distance, time, and speed were log-transformed and gave normally distributed residuals. Carbohydrate contents were square-root transformed. If the ANOVA revealed significant differences between lines or treatments, *post-hoc* testing was performed using Tukey's HSD test. The effect of tetracycline on the flight potential, energy reserves, and wing lengths of the WT and OX3604C lines were analyzed with t-tests.

Results

Flight potential

Males of the WT line spent significantly more time (19–21%) in flight than males of the modified lines ($F = 2.98$, $df = 2$, $p = 0.05$; Table 1). Males of the WT line flew at similar average speeds and covered similar average distances compared to males of the OX3604C line; however, males of the OX513A line proved to be significantly slower flyers (16%; $F = 5.53$, $df = 2$, $p = 0.005$), and covered significantly less distance (38%; $F = 6.07$, $df = 2$, $p = 0.003$; Table 1).

The addition of tetracycline to the diet of WT and OX3604C males did not affect any of the parameters of flight potential that we measured [WT: time $t(35.47) = -0.55$, $p = 0.58$; speed $t(45.53) = 0.82$, $p = 0.41$; distance $t(37.18) = -0.07$, $p = 0.95$; OX3604C: time $t(52.94) = 1.57$, $p = 0.12$; speed $t(50.27) = 0.52$, $p = 0.60$; distance $t(52.99) = 1.99$, $p = 0.06$].

Biochemical analysis

Glycogen ($F = 1.07$, $df = 2$, $p = 0.35$), and sugar ($F = 0.36$, $df = 2$, $p = 0.70$) reserves were similar between the three lines (Table 1). However, OX3604C males had generally higher lipid reserves than males of the other lines ($F = 9.42$, $df = 2$, $p = < 0.001$; Table 1).

The addition of tetracycline to the larval diet of WT and OX3604C males did not influence their glycogen content [WT: $t(47.91) = -0.75$, $p = 0.46$; OX3604C: $t(45.98) = 0.88$, $p = 0.38$]. However, it did increase the sugar [$t(45.34) = 3.85$, $p < 0.001$], and lipid content [$t(42.88) = 9.17$, $p < 0.001$] of the WT males. In the OX3604C males sugar content was not affected [$t(46.98) = -0.02$, $p = 0.99$]; however, lipid content was significantly increased [$t(46.36) = 2.15$, $p = 0.04$].

Wing length

Males of the three lines, reared under the conditions described above, were of similar size ($F = 0.90$, $df = 2$, $p = 0.41$; WT: 2.06 ± 0.01 mm; OX513A: 2.03 ± 0.02 mm; OX3604C: 2.05 ± 0.01 mm). The addition of tetracycline to the larval diet did not affect the wing lengths of the WT [$t(57.92) = 0.85$, $p = 0.40$] or OX3604C [$t(57.95) = 0.19$, $p = 0.42$] males.

Discussion

The experiments revealed statistically significant differences in the flight potential between the genetically-modified lines and their wild-type counterparts, with both strains of genetically-modified males performing to some extent less well than the WT strain. This may indicate fitness deficits in these lines. A possible explanation for the somewhat diminished flight capacity of the genetically-modified mosquitoes is the potentially negative effect of basal or off-target production of the effector tTAV in both the OX513A and OX3604C lines. Any "leakiness" in this system would lead to higher levels of tTAV (and of the effector VP16) in the genetically-modified mosquitoes, which may adversely affect fitness, even though it is expressed only at sub-lethal levels. Such leakiness may be somewhat different between the two transgenic strains. In OX513A, the positive feedback system has no inherent stage- or sex-specificity, and is expected to be activated in adults held off tet. A similar construct (LA656) in Medfly led to a 300-fold accumulation of tTAV mRNA in adults held off tet relative to those on tet (Gong 2005). This was associated with only a modest (13%) reduction in lifespan, but may affect other parameters such as flight ability to a greater or lesser extent. In the OX3604C line, on the other hand, the *AeAct-4* promoter is used in combination with sex-specific alternative splicing to control tTAV production, targeting gene expression only in the indirect flight muscles of females. Consequently, males of this line can survive without the addition of tetracycline to

Table 1. Comparison of Flight Potential (Time Spent in Flight, Flight Speed, and Distance), and Teneral Energy Reserves (Sugar, Glycogen, and Lipid Content) of Males of the WT, OX3604C and OX513A Lines of *Aedes aegypti*

	Time (h)		*Speed (km/h)*		*Distance (km)*		*Glycogen (J)*		*Sugar (J)*		*Lipids (J)*	
WT	3.68 (±0.31)	A	0.68 (±0.03)	A	2.39 (±0.19)	A	2.75 (±0.10)	A	0.16 (±0.02)	A	8.23 (±0.28)	A
OX3604C	2.90 (±0.24)	B	0.72 (±0.03)	A	1.96 (±.14)	A	2.92 (±.10)	A	0.16 (±0.02)	A	9.20 (±0.23)	B
OX513A	2.99 (±0.46)	B	0.57 (±0.04)	B	1.49 (±0.20)	B	2.95 (±0.16)	A	0.16 (±0.04)	A	7.40 (±0.31)	A
	Time (h)		*Speed (km/h)*		*Distance (km)*		*Glycogen (J)*		*Sugar (J)*		*Lipids (J)*	
WT off tet	4.13 (±0.42)	A	0.65 (±0.04)	A	2.58 (±0.24)	A	2.82 (±0.16)	A	0.10 (±0.03)	A	6.66 (±0.17)	A
WT on tet	3.22 (±0.42)	A	0.72 (±0.04)	A	2.20 (±0.24)	A	2.67 (±0.14)	A	0.22 (±0.04)	B	9.80 (±0.30)	B
	Time (h)		*Speed (km/h)*		*Distance (km)*		*Glycogen (J)*		*Sugar (J)*		*Lipids (J)*	
OX3604C off tet	2.68 (±0.38)	A	0.71 (±0.04)	A	1.74 (±0.21)	A	2.85 (±0.14)	A	0.16 (±0.03)	A	8.66 (±0.30)	A
OX3604C on tet	3.17 (±0.41)	A	0.74 (±0.04)	A	2.21 (±0.23)	A	2.99 (±0.13)	A	0.16 (±0.02)	A	9.73 (±0.33)	B

Averages are calculated from the performance of 3-day-old males that covered at least 500 m in a 20-h period. Average lipid and carbohydrate contents were calculated from newly eclosed males (age <2 h). Values denoted by the same letter are not significantly different (95% confidence interval). All values mean (±standard error).

WT, wild-type; J, joules; tet, tetracycline.

their larval diet, and are far less likely to suffer from excess effector production. However, any such effect might predominantly affect the indirect flight muscles—the target tissue in females—and so might have a greater effect on flight ability relative to other fitness parameters. This fits with our findings, as OX3604C males generally compared more favorably to the WT males than did the OX513A males.

Other potential reasons for performance differences between the strains include an effect of the fluorescent marker gene or other components of the transgene; insertional effects of the DNA construct; or differences in strain background (e.g., due to drift, selection, or for OX513A, incomplete exchange of the genetic background when introgressing into the WT strain).

No significant difference in wing length of the males was observed among the three lines, reared either with or without the addition of tetracycline, so the differences between the flight potential and energy content of males were not due to differences in size.

Whatever the exact mechanism involved, the differences in flight performance may need to be taken into account when designing release programs, by positioning release sites appropriately to achieve adequate coverage. Indeed, subsequent to this study, a field mark-release-recapture experiment also revealed somewhat lower dispersal (mean distance traveled) of the OX513A compared with WT line (Lacroix et al. 2011, submitted), consistent with the flight mill data presented here.

The rearing conditions necessary for modified mosquitoes (i.e., the addition of tet to the larval diet), did not affect any parameters of flight that were tested (time spent in flight, flight speed, or distance flown), at least in the two lines WT and OX3604C, for which these comparisons were possible.

In contrast, the amount of energy reserves available to males upon eclosion differed among the three lines and rearing treatments. The addition of tetracycline to the larval diet increased the amount of lipids present upon emergence in both WT and OX3604C males. Furthermore, the sugar contents of WT males almost doubled. The fact that the addition of tet to the larval diet had the most pronounced effect on the WT strain may be because this strain, unlike the genetically-modified lines, is not usually exposed to tet during rearing. It is conceivable that prolonged exposure to such rearing conditions may result in a change in the gut flora of the mosquitoes by selecting for tet-resistant strains of commensals (Kümmerer et al. 2004). These changes might, in turn, effect the metabolic processes of the insects.

Somewhat unexpectedly, the teneral reserves of males did not correlate with their flight potential (i.e., though the lines had similar glycogen and sugar reserves upon emergence, which are the main source of flight fuel, the WT line outperformed the modified lines; Briegel et al. 2001a). As the flight mill trials were conducted on 3-day-old mosquitoes supplied unlimited access to 10% sucrose solution during their maturation, energy levels presumably changed from their state at emergence. This suggests that WT males may use their available energy sources more efficiently after emergence, or that their foraging and feeding behavior differs from those of engineered males.

Sugar may not be as readily available to newly-emerged adults in the field as in our experiments, and thus with regard to release programs of sterile males, it may be beneficial, if practicable, to release adult males after provision of a high-energy sugar meal, rather than at juvenile stages.

The addition of tetracycline led to a significant increase in lipid stores in both the WT and OX3604C line. Tetracycline and tet-based compounds have in the past been added to animal feeds (e.g., to chicken and pig feed), as growth promoters (Swan Report, 1969). In this study, we show for the first time that a tet-based larval feeding substrate significantly increases lipid levels in *A. aegypti*. Though it is difficult to infer differences in field performance from differences in energy reserves, it is conceivable that the increase in lipid stores could give the tet-reared males an advantage after release, since lipid reserves are mobilized rapidly during the first "starving phase" after emergence (Van Handel 1965). However, for female *A. aegypti*, it was shown that mainly carbohydrates were utilized for flying, and that lipid most likely plays a subordinate role as flight fuel (Briegel et al. 2001a). Whether this

holds true for males is not known. Thus our results reveal gaps in our knowledge about this field, and suggest ways to improve mosquito field performance by tailoring rearing conditions to the specific strain.

In conclusion, more extensive comparisons with field-bred mosquitoes remain desirable. The WT line, chosen because of its genetic similarity to the transformed lines, is itself a highly lab-adapted strain, and as such, may offer insight into the effects of genetic manipulation and rearing conditions on flight potential; however, it may not be very representative of field stock. Though the experiments described in this article were not designed to extrapolate dispersal distances in the field, we believe that our controlled and relatively quick approach to evaluating flight potential shows promise as a quality testing tool in transgenic line development.

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Author Disclosure Statement

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